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(54) Title: REC2 KI[illegible]

(54) Titre: KINASE [illegible]

(57) Abstract

The invention [illegible]thod of phosphorylating a serine containing substrate by incubating the substrate with ATP and an enzyme that is hsRec[illegible]ec2 or a derivative thereof. The natural substrates of the kinase activity of Rec2 are the cell cycle control proteins such [illegible]d cyclin E. The over expression of Rec2 is known to cause cell-cycle arrest and apoptosis and the invention discloses tha[illegible]ffects are kinase mediated. Accordingly, the invention provides a method of assessing antagonists and agonists of Rec[illegible]ntagonists and agonists would have pharmacological activity. The invention further discloses that there is specific bin[illegible]n hsRec2 and at least three cell cycle control proteins: p53, PCNA and cdc2.

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L'invention se ra[illegible]un procédé de phosphorylation d'un substrat contenant la sérine, ce procédé étant réalisé par incubation du substr[illegible] de l'ATP et d'une enzyme hsRec2 ou muRec2, ou d'un dérivé de ladite enzyme. Les substrats naturels de l'activité [illegible]Rec2 sont les protéines de régulation du cycle cellulaire, telles que la p53 et la cycline E. La surexpression de R[illegible]outée provoquer l'interruption et l'apoptose du cycle cellulaire, et selon l'invention, ces effets sont liés à la pré[illegible]ase. En conséquence, l'invention porte sur un procédé d'évaluation d'antagonistes et d'agonistes de Rec2 pouvant [illegible]dité pharmacologique. L'invention porte en outre sur la découverte selon laquelle il existe une liaison spécifique e[illegible]t au moins trois protéines de régulation du cycle cellulaire: p53, PCNA et cdc2.

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Description

REC2 KINASE

1. FIELD OF THE INVENTION

The present invention concerns the field of molecular genetics and medicine. Particularly, it concerns a gene encoding a protein that is a kinase and is involved in cell cycle regulation and the repair of damaged genomic DNA in mammalian cells. The gene and protein, termed herein, respectively *hsREC2* and hsRec2, is in the same supergene family as the mammalian protein having homologous pairing and strand transfer activities, *RAD51* and was isolated because of its homology to the homologous pairing and strand transfer protein of *Ustilago maydis*. Due to this relationship the same gene and protein is termed elsewhere *RAD51B* and Rad51B.

2. BACKGROUND OF THE INVENTION

2.1 THE STRUCTURE AND FUNCTION OF *hsREC2*

During the life of every organism the DNA of its cells is constantly subjected to chemical and physical events that cause alterations in its structure, i.e., potential mutations. These potential mutations are recognized by DNA repair enzymes found in the cell because of the mismatch between the strands of the DNA. To prevent the deleterious effects that would occur if these potential mutations became fixed, all organisms have a variety of mechanisms to repair DNA mismatches. In addition, higher animals have evolved mechanisms whereby cells having highly damaged DNA, undergo a process of programmed death ("apoptosis").

The association between defects in the DNA mismatch repair and apoptosis

inducing pathways and the development, progression and response to treatment of oncologic disease is widely recognized, if incompletely understood, by medical scientists. Chung, D.C. & Rustgi, A.K., 1995, Gastroenterology 109:1685-99; Lowe, S.W., et al., 1994, Science 266:807-10. Therefore, there is a continuing need to identify and clone the genes that encode proteins involved in DNA repair and DNA mismatch monitoring.

Studies with bacteria, fungi and yeast have identified three genetically defined groups of genes involved in mismatch repair processes. The groups are termed, respectively, the excision repair group, the error prone repair group and the recombination repair group. Mutants in a gene of each group result in a characteristic phenotype. Mutants in the recombination repair group in yeast result in a phenotype having extreme sensitivity to ionizing radiation, a sporulation deficiency, and decreased or absent mitotic recombination. Petes, T.D., et al., 1991, In Broach, J.R., et al., eds., THE MOLECULAR BIOLOGY OF THE YEAST *SACCHAROMYCES*, pp. 407-522 (Cold Spring Harbor Press, 1991).

Several phylogenetically related genes have been identified in the recombination repair group: *recA*, in *E. Coli*, Radding, C.M., 1989, Biochim. Biophys. Acta 1008:131-145; *RAD51* in *S. cerevisiae*, Shinohara, A., 1992, Cell 69:457-470, Aboussekhra, A.R., et al., 1992, Mol. Cell. Biol. 12:3224-3234, Basile, G., et al., 1992, Mol. Cell. Biol. 12:3235-3246; *RAD57* in *S. cerevisiae*, Gene 105:139-140; *REC2* in *U. maydis*, Bauchwitz, R., & Holloman, W.K., 1990, Gene 90:285-288, Rubin, B.P., et al., 1994, Mol. Cell. Biol. 14:6287-6296. A third *S. cerevisiae* gene *DMC1*, is related to *recA*, although mutants of *DMC1* show defects in cell-cycle progression, recombination and meiosis, but not in recombination repair.

The phenotype of *REC2* defective *U. maydis* mutants is characterized by extreme sensitivity to ionizing radiation, defective mitotic recombination and interplasmid recombination, and an inability to complete meiosis. Holliday, R., 1967, Mutational Research 4:275-288. UmREC2, the *REC2* gene product of *U. maydis*, has been extensively studied. It is a 781 amino acid ATPase that, in the presence of ATP, catalyzes the pairing of homologous DNA strands in a wide

variety of circumstances, e.g., UmREC2 catalyzes the formation of duplex DNA from denatured strands, strand exchange between duplex and single stranded homologous DNA and the formation of a nuclease resistant complex between identical strands. Kmiec, E.B., et al., 1994, Mol. Cell. Biol. 14:7163-7172. UmREC2 is unique in that it is the only eukaryotic ATPase that forms homolog pairs, an activity it shares with the *E. coli* enzyme recA.

U.S. patent application, Serial No. 08/373,134, filed January 17, 1995, by W.K. Holloman and E.B. Kmiec discloses *REC2* from *U. maydis*, methods of producing recombinant UmREC2 and methods of its use. Prior to the date of the present invention a fragment of human *REC2* cDNA was available from the IMAGE consortium, Lawrence Livermore National Laboratories, as plasmid p153195. Approximately 400 bp of the sequence of p153195 had been made publicly available on dbEST database.

The scientific publication entitled: ISOLATION OF HUMAN AND MOUSE GENES BASED ON HOMOLOGY TO REC2, July 1997, Proc. Natl. Acad. Sci. **94**, 7417-7422 by Michael C. Rice et al., discloses the sequences of murine and human Rec2, of the human REC2 cDNA, and discloses that irradiation increases the level of hREC2 transcripts in primary human foreskin fibroblasts. The scientific publication Albala et al., December 1997, Genomics **46**, 476-479 also discloses the sequence of the same protein and cDNA which it terms RAD51B. A sequence that is identical to hsREC2 except for the C-terminal 14 nucleotides of the coding sequence and the 3'-untranslated sequence was published by Cartwright R., et al., 1998, Nucleic Acids Research **26**, 1653-1659 and termed hsR51h2. It is believed that hsREC2 and hsR51h2 represent alternative processing of the same primary transcript. The parent application of this application was published as WO 98/11214 on March 19, 1998.

The structure of hsREC2 is also disclosed in application Serial No. 60/025,929, filed September 11, 1996, application Serial No. 08/927,165, filed September 11, 1997, and patent publication WO 98/11214, published March 19, 1998.

2.2 CELL CYCLE REGULATION

The eukaryotic cell cycle consists of four stages, G_1, S (synthesis), G_2, and M (mitosis). The underlying biochemical events that determine the stage of the cell and the rate of progression to the next stage is a series of kinases, e.g., cdk2, cdc2, which are regulated and activated by labile proteins that bind them, termed cyclins, e.g., cyclin D, cyclin E, Cyclin A . The activated complex in turn phosphorylates other proteins which activates the enzymes that are appropriate for each given stage of the cycle. Reviewed, Morgan, D.O., 1997, Ann. Rev. Cell. Dev. Biol. **15**, 261-291; Clurman, B.E., & Roberts, J.M., 1998, in THE GENETIC BASIS OF HUMAN CANCER, pp.173-191 (ed. by Vogelstein, B., & Kinzer K.W., McGraw Hill, NY) (hereafter *Vogelstein*)

The cell cycle contains a check point in G_1. Under certain conditions, e.g., chromosomal damage or mitogen deprivation, a normal cell will not progress beyond the check point. Rb and p53 are proteins involved in the G_1 check point related to mitogen deprivation and chromosomal damage, respectively. Inactivating mutations in either of these proteins results, in concert with other mutations, in a growth transformed, i.e., malignant, cell. The introduction of a copy of the normal p53 or Rb gene suppresses the transformed phenotype. Accordingly genes, such as p53 or Rb, whose absence is associated with transformation are termed "tumor suppressor" genes. A frequent cause of familial neoplastic syndromes is the inheritance of a defective copy of a tumor suppressor gene. Reviewed Fearson, E.R., in *Vogelstein* pp. 229-236.

The level of p53 increases in response to chromosomal damage, however, the mechanism which mediates this response is unknown. It is known that p53 can be phosphorylated by a variety of kinases and that such phosphorylation may stabilize the p53 protein. Reviewed Agarwal, M.L., et al., Jan. 2,1998, J. Biol. Chem. 273, 1-4.

3. SUMMARY OF THE INVENTION

The present invention is based on the unexpected discovery that hsRec2 is a serine kinase that phosphorylates several proteins that control the cell cycle, particularly cyclin E and p53. The invention permits the phosphorylation of the cell cycle control proteins at sites that are physiologically elevant. In addition, the discovery of the enzyme activity of Rec2 permits the construction of assays for the discovery of compounds that are specific antiagonists and agonists of Rec2, which compounds have a pharmacological activity.

4. BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-1D.

Figures 1A and 1B show the derived amino acid sequence of hsREC2 (SEQ ID NO:1) and Figures 1C and 1D show the nucleic acid sequences of the *hsREC2* cDNA coding strand (SEQ ID NO:2). Figures 1E and 1F show the derived amino acid sequence of muREC2 (SEQ ID NO:3) and Figure 1G shows the nucleic acid sequences of the *muREC2* cDNA coding strand (SEQ ID NO:4).

Figure 2A-2C.

Figure 2A is an annotated amino acid sequence of hsREC2. Specifically noted are the nuclear localization sequence ("NLS"), A Box and B Box motif sequences, DNA binding sequence and a src-type phosphorylation site ("P"). Figure 2B is a cartoon of the annotated sequence, showing in particular that the region 8-200 is most closely related to recA. Figures 2C and 2D show the sequence homology between hsREC2 and *Ustilago maydis* REC2. The region of greatest similarity, 43% homology, is in bold.

Figure 3A-3B.

3A. The incorporation of ^{32}P-ATP into myelin basic protein (0.25 μM) as a function of time, concentration of Rec2 was 1 μg/30-40μl. 3B. The incorporation of ^{32}P-ATP into kemptide (LRRASLG, SEQ ID No: 5) during a 60 min. reaction as a function of kemptide concentration.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, genes are all capitlized , e.g., hsREC2, while the corresponding protein is in initial capitalization, e.g., hsRec2.

The activity of hsREC2 was determined using an N-terminal hexahistadyl containing derivative that was produced in baculovirus. Confirming results were obtained with baculovirus produced glutathione-S-transferase conjugated hsREC2 and with thioredoxin-conjugated hsREC2 produced in *E. coli*. These confirming results tend to exclude that the kinase activity resulted from the co-purification of an endogenous baculovirus kinase on the Ni-NTA resin. To further exclude the possibility of purification artifacts the Ni-NTA purified hexahistadyl-hsREC2 was further purified by preparative SDS-PAGE. Only the fractions containing hsREC2 by silver stain were found to contain kinase activity.

The sequence of muRec2 and hsRec2 differ at only 56 of the 350 amino acids. The invention can be practiced using either muRec2 or hsRec2 or a protein that consists of a mixture of amino acids, i.e., at some positions the amino acid is that of muRec2 and at others the amino acid is that of hsRec2, hereafter a chimeric hs/muRec2. In addition, the mutein having a substitution for the tyrosine at position 163 can be used to practice the invention, e.g., Tyr→Ala . Thus, the invention can be further practiced using a chimeric hs/muREC2^{ala163}. In one embodiment the substitution can be any aliphatic amino acid. In an alternative embodiment the substitution can be any amino acid other than cysteine or proline. The term "Rec2 kinase" is used herein to denote the genus consisting of hsRec2, muRec2 and all chimeric hs/muRec2 proteins and the Tyr^{163} substituted derivatives of each. The term artificial Rec2 kinase is a Rec2 kinase that is not also a mammalian Rec2. The term mammalian Rec2 is used herein to denote the genus of proteins consisting of the mammalian homologs of hsRec2 and of muRec2.

The invention can further be practiced using a fusion protein, which consists of a protein having a sequence that comprises that of a Rec2 kinase or a mammalian Rec2 that is fused to a second sequence which is a protein or peptide that can be used to purify the resultant fusion protein.

The naturally occurring hsRec2 and muRec2 are found as phosphoproteins, the phosphorylation of which is not essential to the activity of the proteins as a kinase. In the invention the terms Rec2 kinase and mammalian Rec2 encompass both the phosphorylated and non-phosphorylated forms of the proteins.

Cell Cycle Regulation

An expression vector comprising hsREC2 operably linked to the CMV immediate early promoter was constructed and transfected into CHO cells. A mutein was constructed in which tyrosine-163, a phosphorylatable tyrosine in an *src* site (glu-pro-arg-tyr) (amino acids 8-11 of SEQ ID No. 8) was replaced by alanine ($hsREC2^{ala163}$). Sham (neo^r) transfected, hsREC2 transfected and $hsREC2^{ala163}$ transfected CHO cells were synchronized by serum starvation, released, and the DNA content was assayed by quantitative fluorescent flow cytometry at various time points. The hsREC2 transfected cells showed delayed onset of S phase. Thus, at 14 hours post release 75% of the hsREC2 transfected cells were in G_1 compared to 36% of the controls.

Over expression of hsREC2 but not $hsREC2^{ala163}$ sensitizes the cell to UV radiation. CHO cells were irradiated with UV at 15 J/m^2. Again the cells were analyzed by quantitative fluorescent flow cytometry The hsREC2 cells showed extensive apoptosis compared to the controls at 24, 48 and 72 hours post irradiation.

Kinase Activity

The kinase activity of hsREC2 can be shown on a variety of substrates. Artifactual substrates such as myelin basic protein, which is a known substrate for protein kinase C and protein kinase A are phosphorylated by hsREC2. The kemptide (leu-arg-arg-ala-ser-leu-gly), which is also a known substrate of ser/thr kinases can be phosphorylated. In addition the following recombinantly produced proteins are phosphorylated by hsREC2: p53, cyclin B1 and cyclin E. The heterodimers of cyclin B1/cdc2 and cyclin E/cdk2 are also phosphorylated by hsREC2. The interpretation of these experiments is complicated by the fact that

cyclin E/cdk2 autophosphorylates and that cyclin B1/cdc2 but not cyclin E/cdk2 phosphorylates hsREC2 itself. In contrast to the cyclinB1/cdc2 complex, hsRec2 is not an autophosphorylase.

Although expression of hsREC2^{ala163} in a cell has no effect on the cell cycle, the hsREC2^{ala163} mutein has full kinase activity.

Compounds having pharmacological activity with respect to mREC2 can be identified by assaying the kinase activity of an mREC2, and particularly hsREC2, in the presence of candidate agonists or antagonists. The particular preferred substrates are cyclin E and p53.

hsREC2 Association With Other Proteins

A S^{35} -radiolabeled preparation of hsREC2 was made by coupled transcription translation in a recticulocyte lysate system. The preparation was mixed with an extract from HCT116 cells. In separate reactions monoclonal antibodies to various cell proteins were added and the antibody bound material isolated with Protein A Sepharose. The bound material was then analyzed by SDS-PAGE and autoradiographed. The immunoprecipitate contained hsREC2 when anti-p21, anti-PCNA and anti-cdc2 monoclonals were used. No hsREC2 was precipitated when anti-cdc4 or anti-cdk4 monoclonals were employed.

An hsREC2 Agonist or Antagonist Has a Pharmacologic Activity

The activities of hsREC2 indicate that the modulation of its activity can sensitize or desensitize a cell to enter apoptosis as a result of incurring genetic damage, as for example by UV radiation, and can also protect or deprotect a cell from DNA damage by extending or shortening the G_1 and S periods. Agonist and antagonists of hsREC2 are compounds having activities of the type that medical practitioners desire. The discovery of compounds that are hsREC2 agonists or antagonists will be important in pharmaceutical science.

In one embodiment, the invention is a method of determining whether a given compound has such a pharmacological activity by measuring the effects of

the compound on the kinase activity of hsREC2. In specific embodiments, the invention is a method wherein the relative effects of the compound on hsREC2 and on a second kinase are assessed. For example, a compound that is an agonist of hsREC2 but has little or no effect on cyclin D/cdk4 and cyclin E/cdk2 would cause cells to arrest in G_1 and undergo apoptosis in response to genetic damage. In particular embodiments, the kinase assay is done with a substrate that is selected from the group consisting of p53, cdc2, cdk2 or cyclin E. Alternatively, the substrate can be a model substrate such as myelin basic protein or kemptide (leu-arg-arg-ala-ser-leu-gly).

6. EXAMPLES

6.1 The production of recombinant hsREC2 protein by baculovirus infection of *Autographica californica*

To facilitate the construction of an *hsREC2* expression vector, restriction sites for XhoI and KpnI were appended by PCR amplification to a the *hsREC2* cDNA. The *hsREC2* cDNA starting at nt 71 was amplified using the forward primer 5' GAG CTCGAG GGTACC C ATG GGT AGC AAG AAA C-3' (SEQ ID NO:7) which placed the XhoI and KpnI sites (underlined) 5' of the start codon. The recombinant molecule containing the entire coding sequence of *hsREC2* cDNA, could be removed using either XhoI or KpnI and the unique XbaI site located between nt 1270 and 1280 of SEQ ID NO:2.

A vector, pBacGSTSV, for the expression of HsREC2 in baculovirus infected *Spodoptera frugiperda* (Sf-9) insect cells (ATCC cell line No. CRL1711, Rockville, MD), was obtained from Dr. Zailin Yu (Baculovirus Expression Laboratory, Thomas Jefferson University). The vector pVLGS was constructed by the insertion of a fragment encoding a *Schistosoma japonicum* glutathione S-transferase polypeptide and a thrombin cleavage site from pGEX-2T (described in Smith & Johnson, GENE 67:31 (1988)), which is hereby incorporated by reference, into the vector pVL1393. A polyA termination signal sequence was inserted into pVLGS to yield pBacGSTSV. A plasmid containing the 1.2 Kb *hsREC2* fragment was cut with KpnI, the 3' unpaired ends removed with T4

polymerase and the product cut with XbaI. The resultant fragment was inserted into a-SmaI, Xbal cut pBacGSTSV vector to yield pGST/hsREC2.

Recombinant virus containing the insert from pGST/hsREC2 were isolated in the usual way and Sf-9 cells were infected. Sf-9 cells are grown in SF900II SFM (Gibco/BRL Cat # 10902) or TNM-FH (Gibco/BRL Cat # 11605-011) plus 10% FBS. After between 3-5 days of culture the infected cells are collected, washed in Ca^{++} and Mg^{++} free PBS and sonicated in 5ml of PBS plus protcinase inhibitors (ICN Cat # 158837), 1% NP-40, 250 mM NaCl per $5x10^7$ cells. The lysate is cleared by centrifugation at 30,000 xg for 20 minutes. The supernatant is then applied to 0.5 ml of glutathione-agarose resin (Sigma Chem. Co. Cat # G4510) per $5x10^7$ cells. The resin is washed in a buffer of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl and 2.5 mM $CaCl_2$, and the hsREC2 released by treatment with thrombin (Sigma Chem. Co. Cat # T7513) for 2 hours at 23°C in the same buffer. For certain experiments the thrombin is removed by the technique of Thompson and Davie, 1971, Biochim Biophys Acta 250:210, using an aminocaproyl-p-chlorobenzylmide affinity column (Sigma Chem. Co. Cat # A9527).

Alternatively, the full length hsREC2 cDNA was cloned into the expression vector, pAcHisA, for overexpression in a baculovirus system and purification utilizing a 6 histidine tag. For cloning, the hsREC2 expression cassette was cut with KpnI, the 3' protruding termini were removed with T4 polymerase, and the DNA was then digested with XbaI. The resulting fragment was ligated to pAcHisA using the SmaI and XbaI sites. Recombinant virus containing hsREC2 was purified and insect cells were infected by Dr. Z. Yu in the Baculovirus expression laboratory of the Kimmel Cancer Institute. Insect cell pellets from 2 liters of culture were suspended in 60 ml of 10 mM TrisCl, pH 7.5, 130 mM NaCl, 2% TX100, 2 μg/ml leupeptin and aprotinin and 1 μg/ml pepstatin and sonicated on ice 4 times for 5 seconds each using a microtip at a 20% pulse (Branson sonifier 450). Debris was removed by centrifuging at 30,000 xg for 20 minutes. The clarified supernatant was divided between two 50 ml culture tubes and 1 ml of Ni-NTA agarose added to each tube for 1 hour with rocking at 4°C. The unbound fraction was separated from

the resin by a brief centrifugation and the resin was washed with 10 ml of 100 mM imidazole for 10 minutes on a rocker and centrifuged at 2000 rpm for 5 minutes. After a second 10 minute wash with 500 mM imidazole the slurry was transferred to a column and the effluent discarded. The purified his-hsRec2 was eluted with 1M imidazole, pH 7.0 (imidazole on column for 10 minutes before collection of eluate), and dialyzed overnight against 50 mM TrisCl, pH 7.4, 50 mM NaCl, 10% glycerol. For simplicity, this protein will be referred to as hsRec2 instead of hishsRec2.

6.2 **The Bacterial Production of recombinant hsREC2 protein**

The hsREC2 cDNA coding region was excised from the previously used mammalian expression vector pcDNA3 G8 by cleavage with XbaI, removal of 5' protruding termini with T4 polymerase, followed by cleavage with KpnI. The resulting fragment was ligated into the KpnI and blunted HindIII sites of a bacterial expression vector pBAD/HisC (Invitrogen, Corp., USA). The constructed expression vector with hREC2 cloned in frame with a hexahistidine tag was electrotransformed into LMG194 bacteria (Invitrogen, Corp., USA) for expression. A 500ml LB ampicillin culture was inoculated by a single colony and grown at 37° into log phase. The culture was induced by .02% arabinose for 4 hours and harvested by centrifuging at 8,000 xg. The pellet was resuspended and lysed by 1mg/ml lysozyme and sonication in 5 volumes of 50mM NaH_2PO_4, 300mM NaCl, 1% TX100, 2μg/ml leupeptin and aprotinin and 1μg/ml pepstatin, .1 mg/ml DNase I, 10mM βME and 20mM imidazole at 0°C. The lysate was clarified by centrifugation at 10,000 xg for 30 minutes then added to a sealed column containing 1 ml activated Ni+NTA agarose resin and rocked at 4 for 1 hour. The column was then opened and washed by gravity with 20 volumes of 50mM NaH_2PO_4, 300mM NaCl, 1% TX100, 50mM imidazole at 4°. The bound protein was then eluted in 3 volumes of the above wash buffer with 500mM imidazole and collected in 1ml fractions. The purified His-HsRec2 was dialyzed over night against 50mM Tris, 50mM NaCl, 10% glycerol and stored at -80°.

6.3 Detection of hsREC2 Kinase

Phosphokinase filter assays. Substrates were either kemptide or myelin basic protein and approximately 1 μg of his-hsRec2 was added as the phosphokinase. For both assays, the buffer contained 50 mM TrisCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT. The second substrate, ^{32}P-ATP was constant at 50 μM with a specific activity of 1972 cpm/pmole (kemptide) and 2980 cpm/pmole (MBP). ^{32}P-ATP was added to initiate the reaction which was carried out at 30° C. for the indicated time. At the end of the reaction, 20 μl was spotted on phosphocellulose discs, washed twice with 10 ml per disc in 1% phosphoric acid and twice in distilled water. Filters were counted in a Wallac Scintillation counter. Substrate without hsRec2 added was used as a control and counts were subtracted to obtain a zero point.

Myelin basic protein (0.25 μM) was phosphorylated for between 0 and 25 minutes at the above conditions. Phosphate incorporation was linear with time and reached 1.2 pmole at 25 minutes. Kemptide from 0 to 0.15 mM was phosphorylated for 60 minutes. The rate of phosphate incorporation was linear with substrate concentration up to 0.06 mM, where a rate of 0.09 pmoles/minute was observed.

Two different hsRec2 conjugates, GST-hsRec2 and thioredoxin-hsRec2, also exhibited phosphokinase activity. Further evidence that this activity was not a contaminant, was obtained by immunoprecipitating hsREC2 using hybridoma supernatants, followed by assay for phosphokinase activity using p53 as a substrate as described below. These experiment confirmed that the kinase activity was precipitable by anti-hsREC2 monoclonal antibodies.

Two substrates that were not phosphorylated by hsRec2, were a tyrosine kinase substrate peptide containing one tyrosine, derived from the sequence surrounding the phosphorylation site in pp60^{src} (RRLIEDAEYAARG) (SEQ ID No. 7), and an hsRec2 peptide, residues 153-172 (VEIAESRFPRYFNYEEKLLL) (SEQ ID No. 8).

p53 phosphorylation. Human recombinant p53 (0.5 μg, Pharmingen, San Diego, CA) was incubated with or without hsRec2 in 50 mM TrisCl, pH 7.4,

10 mM $MgCl_2$, and 1 mM DTT at 30° C. The reaction was initiated by the addition of ^{32}P-ATP (25 μM ATP, 40 cpm/femtomole). At the end of each time point an equal volume of 2X loading buffer (5) was added and tubes were placed on ice until all tubes were collected. Samples were then heated at 100° C for 10 minutes and 13 μl were run on Ready Gels (Bio-Rad Laboratories, Hercules, CA), and transferred to nitrocellulose overnight prior to exposure to X-ray film. Radiolabeled p53 was readily observed.

cdc2/cyclin B phosphokinase assay. Purified human recombinant cyclin B1/cdc2 (Oncogene, Cambridge, MA), was incubated with hsRec2 for 10 or 60 minutes at 30° C., using the same buffer conditions as described for p53. An equal volume of 2X gel lading buffer was added (5), samples were heated at 100° C for 10 minutes and run on an SDS gel, transferred to nitrocellulose and exposed to film. Radiolabeled cyclin B1 due to hsREC2 kinase activity was readily observed above the level of "autophosphorylation" of cyclin B1 by cdc2. Radiolabeled cdc2 was observed only in the hsREC2 containing reactions mixtures at 60 minutes but not at 10 minutes reaction time.

cdk2/cyclin E phosphokinase assay. GST-cyclin E was isolated from *E. coli* transformed with pGEX-2TcycE (A. Giordano, Thomas Jefferson University) and purified using Glutathione Sepharose 4B (Pharmacia, Piscataway, NJ). The glutathione Sepharose GST-cyclin E was washed, and then stored as a 1:1 slurry in 50 mM Tris Cl, pH 7.4. For assays with cyclin E bound cdk2, purified cdk2 (kindly given to us by A. Koff, Sloan-Kettering, NY) was incubated with cyclin E as described (6) and unbound cdk2 removed by washing prior to storage as a 1:1 slurry. Kinase assays were carried out with the immobilized GST-cyclin E with or without bound cdk2 otherwise using the same conditions described for p53. Phosphorylation of cyclin E and hsREC2 was readily observed in the absence of cdk2. In the presence of cdk2, autophosphorylation was seen, however hsREC2 phosphorylation of cyclin E above that level was readily apparent.

***In vitro* associated between p53 and hsRec2.** HsRec2 (5 μg) and 15 μl agarose GST-p53 (Oncogene Sciences) were added to 0.5 ml of binding buffer

(10%) glycerol, 50 mM Tris Cl, pH 7.4, 0.1 mM EDTA, 1mM DTT, 0.02% NP40, 200mM NaCl, 10μg/ml aprotinin and leupeptin , and 20 μM PMSF. Following one hour at room temperature, the p53 agarose was pelleted and washed twice with buffer as above, using a higher concentration of detergent (0.1% NP40), and once with 50mM TrisCl, pH 7.4, 10mM $MgCl_2$.

Association of *in vitro* translated hsRec2 with PCNA, p53 and cdc2. XbaI linearized pCMVhREC2 was first transcribed in vitro (Ambion, Austin TX) using 1 μg of the vector, and then translated *in vitro* along with Xef1 mRNA included in the kit as a positive control. Reticulocyte lysates containing Xef1 or hsRec2 translation products labeled with ^{35}S-methionine were incubated with 1.2 mg cell extract from HCT116 cells (50 mM TrisCl, pH 7.4, 120 mM NaCl, 0.5% NP40, 20 μM PMSF, 2 μg/ml pepstatin, and 10 μg/ml leupeptin and aprotinin, MB) for 2 hours, then 10 μg of antibodies against PCNA, p53 or cdc2 were added for an overnight incubation. On the following day, Protein A Sepharose was added for 2 hours, and pellets were washed four times with 500 μl MB. Pellets were suspended in 40 μl of sample buffer, boiled 10 minutes and 15 μl run on a 10% gel, then transferred to nitrocellulose to obtain a lower background, before exposure to X-ray film.

Claims

CLAIMS:

1. A method of phosphorylating a serine-containing substrate which comprises incubating the substrate with an effective concentration of ATP and an enzyme having a sequence which comprises the sequence of a Rec2 kinase or a mammalian Rec2 and measuring the amount of phosphorylation of the substrate.

2. The method of claim 1, wherein the sequence of the enzyme comprises the sequence of a Rec2 kinase containing other than a Tyr^{163}.

3. The method of claim 2, wherein the sequence of the enzyme comprises the sequence of hsRec2 containing other than a Tyr^{163}.

4. The method of claim 3, wherein the substrate is selected from the group consisting of the human proteins p53, cdc2, cdk2 and cyclin E.

5. The method of claim 3, wherein the substrate is a kemptide.

6. The method of claim 1, wherein the sequence of the enzyme comprises the sequence of hsRec2.

7. The method of claim 6, wherein the substrate is selected from the group consisting of p53, cdc2, cdk2 or cyclin E.

8. The method of claim 6, wherein the substrate is a kemptide.

9. The method of claim 1, wherein the sequence of the enzyme comprises the sequence of a mammalian Rec2.

10. The method of claim 9, wherein the substrate is selected from the group consisting of the human proteins p53, cdc2, cdk2 and cyclin E.

11. The method of claim 9, wherein the substrate is a kemptide.

12. The method of claim 1, which further comprises the steps of forming a mixture of the enzyme and an antagonist or an agonist of the enzyme and measuring the effect of said antagonist or agonist on the amount of phosphorylation on the substrate.

13. A composition comprising

a. an enzyme having a sequence that comprises the sequence of a Rec2 kinase or a mammalian Rec2;

b. a serine-containing substrate of the enzyme; and

c. a γ-phosphate labeled ATP.

14. The composition of claim 13, in which the labeled phosphate is a ^{32}P.

15. The composition of claim 13, in which the substrate is a cell-cycle control protein.

16. The composition of claim 15 in which the substrate is a protein selected from the group consisting of human p53, human cdc2, human cdk2 and human cyclin E.

17. The composition of claim 13, in which the substrate is a kemptide.

18. The composition of claim 13, in which the sequence of the enzyme comprises the sequence of hsRec2 or hsRec2^{Ala163}.

19. An enzyme comprising a Rec2 kinase having an amino acid that is other than a Tyr^{163}.

20. An enzyme having a sequence comprising the sequence of a mammalian Rec2 having an amino acid that is other than a Tyr^{163}.

```
Met Gly Ser Lys Lys Leu Lys Arg Val Gly Leu Ser Gln Glu Leu Cys
 1               5                  10                  15
Asp Arg Leu Ser Arg His Gln Ile Leu Thr Cys Gln Asp Phe Leu Cys
            20                  25                  30
Leu Ser Pro Leu Glu Leu Met Lys Val Thr Gly Leu Ser Tyr Arg Gly
        35                  40                  45
Val His Glu Leu Leu Cys Met Val Ser Arg Ala Cys Ala Pro Lys Met
    50                  55                  60
Gln Thr Ala Tyr Gly Ile Lys Ala Gln Arg Ser Ala Asp Phe Ser Pro
65                  70                  75                  80
Ala Phe Leu Ser Thr Thr Leu Ser Ala Leu Asp Glu Ala Leu His Gly
                85                  90                  95
Gly Val Ala Cys Gly Ser Leu Thr Glu Ile Thr Gly Pro Pro Gly Cys
            100                 105                 110
Gly Lys Thr Gln Phe Cys Ile Met Met Ser Ile Leu Ala Thr Leu Pro
        115                 120                 125
Thr Asn Met Gly Gly Leu Glu Gly Ala Val Val Tyr Ile Asp Thr Glu
    130                 135                 140
Ser Ala Phe Ser Ala Glu Arg Leu Val Glu Ile Ala Glu Ser Arg Phe
145                 150                 155                 160
Pro Arg Tyr Phe Asn Thr Glu Glu Lys Leu Leu Leu Thr Ser Ser Lys
                165                 170                 175
```

FIG. 1A

```
Val His Leu Tyr Arg Glu Leu Thr Cys Asp Glu Val Leu Gln Arg Ile
            180                 185                 190
Glu Ser Leu Glu Glu Glu Ile Ile Ser Lys Gly Ile Lys Leu Val Ile
        195                 200                 205
Leu Asp Ser Val Ala Ser Val Val Arg Lys Glu Phe Asp Ala Gln Leu
    210                 215                 220
Gln Gly Asn Leu Lys Glu Arg Asn Lys Phe Leu Ala Arg Glu Ala Ser
225                 230                 235                 240
Ser Leu Lys Tyr Leu Ala Glu Glu Phe Ser Ile Pro Val Ile Leu Thr
                245                 250                 255
Asn Gln Ile Thr Thr His Leu Ser Gly Ala Leu Ala Ser Gln Ala Asp
            260                 265                 270
Leu Val Ser Pro Ala Asp Asp Leu Ser Leu Ser Glu Gly Thr Ser Gly
        275                 280                 285
Ser Ser Cys Val Ile Ala Ala Leu Gly Asn Thr Trp Ser His Ser Val
    290                 295                 300
Asn Thr Arg Leu Ile Leu Gln Tyr Leu Asp Ser Glu Arg Arg Gln Ile
305                 310                 315                 320
Leu Ile Ala Lys Ser Pro Leu Ala Pro Phe Thr Ser Phe Val Tyr Thr
                325                 330                 335
Ile Lys Glu Glu Gly Leu Val Leu Gln Ala Tyr Gly Asn Ser
            340                 345                 350
```

FIG. 1B

```
CGGACGCGTG GGCGCGGGGA AACTGTGTAA AGGGTGGGGA AACTTGAAAG TTGGATGCTG   60
CAGACCCGGC ATGGGTAGCA AGAAACTAAA ACGAGTGGGT TTATCACAAG AGCTGTGTGA  120
CCGTCTGAGT AGACATCAGA TCCTTACCTG TCAGGACTTT TTATGTCTTT CCCCACTGGA  180
GCTTATGAAG GTGACTGGTC TGAGTTATCG AGGTGTCCAT GAACTTCTAT GTATGGTCAG  240
CAGGGCCTGT GCCCCAAAGA TGCAAACGGC TTATGGGATA AAAGCACAAA GGTCTGCTGA  300
TTTCTCACCA GCATTCTTAT CTACTACCCT TTCTGCTTTG GACGAAGCCC TGCATGGTGG  360
TGTGGCTTGT GGATCCCTCA CAGAGATTAC AGGTCCACCA GGTTGTGGAA AAACTCAGTT  420
TTGTATAATG ATGAGCATTT TGGCTACATT ACCCACCAAC ATGGGAGGAT TAGAAGGAGC  480
TGTGGTGTAC ATTGACACAG AGTCTGCATT TAGTGCTGAA AGACTGGTTG AAATAGCAGA  540
ATCCCGTTTT CCCAGATATT TTAACACTGA AGAAAAGTTA CTTTTGACAA GTAGTAAAGT  600
TCATCTTTAT CGGGAACTCA CCTGTGATGA AGTTCTACAA AGGATTGAAT CTTTGGAAGA  660
AGAAATTATC TCAAAAGGAA TTAAACTTGT GATTCTTGAC TCTGTTGCTT CTGTGGTCAG  720
AAAGGAGTTT GATGCACAAC TTCAAGGCAA TCTCAAAGAA AGAAACAAGT TCTTGGCAAG  780
AGAGGCATCC TCCTTGAAGT ATTTGGCTGA GGAGTTTTCA ATCCCAGTTA TCTTGACGAA  840
TCAGATTACA ACCCATCTGA GTGGAGCCCT GGCTTCTCAG GCAGACCTGG TGTCTCCAGC  900
```

FIG. 1C

```
TGATGATTTG TCCCTGTCTG AAGGCACTTC TGGATCCAGC TGTGTGATAG CCGCACTAGG    960
AAATACCTGG AGTCACAGTG TGAATACCCG GCTGATCCTC CAGTACCTTG ATTCAGAGAG   1020
AAGACAGATT CTTATTGCCA AGTCCCCTCT GGCTCCCTTC ACCTCATTTG TCTACACCAT   1080
CAAGGAGGAA GGCCTGGTTC TTCAAGCCTA TGGAAATTCC TAGAGACAGA TAAATGTGCA   1140
AACCTGTTCA TCTTGCCAAG AAAAATCCGC TTTTCTGCCA CAGAAACAAA ATATTGGGAA   1200
AGAGTCTTGT GGTGAAACAC CCATCGTTCT CTGCTAAAAC ATTTGGTTGC TACTGTGTAG   1260
ACTCAGCTTA AGTCATGGAA TTCTAGAGGA TGTATCTCAC AAGTAGGATC AAGAACAAGC   1320
CCAACAGTAA TCTGCATCAT AAGCTGATTT GATACCATGG CACTGACAAT GGGCACTGAT   1380
TTGATACCAT GGCACTGACA ATGGGCACAC AGGGAACAGG AAATGGGAAT GAGAGCAAGG   1440
GTTGGGTTGT GTTCGTGGAA CACATAGGTT TTTTTTTTTA ACTTTCTCTT TCTAAAATAT   1500
TTCATTTTGA TGGAGGTGAA ATTTATATAA GATGAAATTA ACCATTTTAA AGTAAACAAT   1560
TCCGTGGCAA CTAGATATCA TGATGTGCAA CCAGCATCTC TGTCTAGTTC CCAAATATTT   1620
CATCACCCCC AAAAGCAAGA CCCATAACCA TTATGCAAGT GTTCCTATTT CCCCCTCCTC   1680
CCAGCTCCTG GGAAACCACC AATCTACTTT TTTTCTATGG CTTTACCTAA TCTGGAAATT   1740
TCAAATAAAT GGGATCAAAT AGTTTCCCAA AAAAAAAAAA AAAAAAAAAA AAAAAAA      1797
```

FIG. 1D

```
Met Ser Ser Lys Lys Leu Arg Arg Val Gly Leu Ser Pro Glu Leu Cys
 1               5                  10                  15
Asp Arg Leu Ser Arg Tyr Leu Ile Val Asn Cys Gln His Phe Leu Ser
            20                  25                  30
Leu Ser Pro Leu Glu Leu Met Lys Val Thr Gly Leu Ser Tyr Arg Gly
        35                  40                  45
Val His Glu Leu Leu His Thr Val Ser Lys Ala Cys Ala Pro Gln Met
    50                  55                  60
Gln Thr Ala Tyr Glu Leu Lys Thr Arg Arg Ser Ala His Leu Ser Pro
65                  70                  75                  80
Ala Phe Leu Ser Thr Thr Leu Cys Ala Leu Asp Glu Ala Leu His Gly
                85                  90                  95
Gly Val Pro Cys Gly Ser Leu Thr Glu Ile Thr Gly Pro Pro Gly Cys
            100                 105                 110
Gly Lys Thr Gln Phe Cys Ile Met Met Ser Val Leu Ala Thr Leu Pro
        115                 120                 125
Thr Ser Leu Gly Gly Leu Glu Gly Ala Val Val Tyr Ile Asp Thr Glu
    130                 135                 140
Ser Ala Phe Thr Ala Glu Arg Leu Val Glu Ile Ala Glu Ser Arg Phe
145                 150                 155                 160
Pro Gln Tyr Phe Asn Thr Glu Glu Lys Leu Leu Leu Thr Ser Ser Arg
                165                 170                 175
```

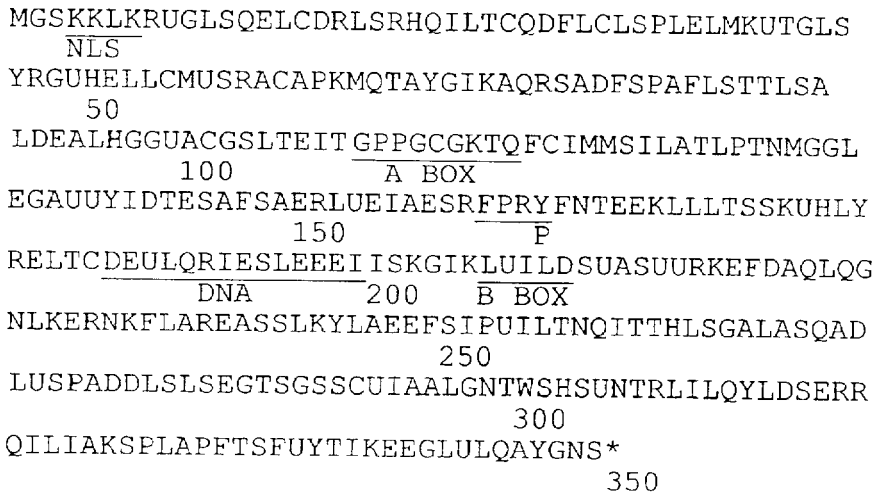
FIG. 1E

```
Val His Leu Cys Arg Glu Leu Thr Cys Glu Gly Leu Leu Gln Arg Leu
                180                 185                 190
Glu Ser Leu Glu Glu Glu Ile Ile Ser Lys Gly Val Lys Leu Val Ile
        195                 200                 205
Val Asp Ser Ile Ala Ser Val Val Arg Lys Glu Phe Asp Pro Lys Leu
    210                 215                 220
Gln Gly Asn Ile Lys Glu Arg Asn Lys Phe Leu Gly Lys Gly Ala Ser
225                 230                 235                 240
Leu Leu Lys Tyr Leu Ala Gly Glu Phe Ser Ile Pro Val Ile Leu Thr
                245                 250                 255
Asn Gln Ile Thr Thr His Leu Ser Gly Ala Leu Pro Ser Gln Ala Asp
            260                 265                 270
Leu Val Ser Pro Ala Asp Asp Leu Ser Leu Ser Glu Gly Thr Ser Gly
        275                 280                 285
Ser Ser Cys Leu Val Ala Ala Leu Gly Asn Thr Trp Gly His Cys Val
    290                 295                 300
Asn Thr Arg Leu Ile Leu Gln Tyr Leu Asp Ser Glu Arg Arg Gln Ile
305                 310                 315                 320
Leu Ile Ala Lys Ser Pro Leu Ala Ala Phe Thr Ser Phe Val Tyr Thr
                325                 330                 335
Ile Lys Gly Glu Gly Leu Val Leu Gln Gly His Glu Arg Pro
            340                 345                 350
```

FIG. 1F

```
GGGAGCCCTG GAAACATGAG CAGCAAGAAA CTAAGACGAG TGGGTTTATC TCCAGAGCTG      60
TGTGACCGTT TAAGCAGATA CCTGATTGTT AACTGTCAGC ACTTTTTAAG TCTCTCCCCA     120
CTAGAACTTA TGAAAGTGAC TGGCCTGAGT TACAGAGGTG TCCACGAGCT TCTTCATACA     180
GTAAGCAAGG CCTGTGCCCC GCAGATGCAA ACGGCTTATG AGTTAAAGAC ACGAAGGTCT     240
GCACATCTCT CACCGGCATT CCTGTCTACT ACCCTGTGCG CCTTGGATGA AGCATTGCAC     300
GGTGGTGTGC CTTGTGGATC TCTCACAGAG ATTACAGGTC CACCAGGTTG CGGAAAAACT     360
CAGTTTTGCA TAATGATGAG TGTCTTAGCT ACATTACCTA CCAGCCTGGG AGGATTAGAA     420
GGGGCTGTGG TCTACATCGA CACAGAGTCT GCATTTACTG CTGAGAGACT GGTTGAGATT     480
GCGGAATCTC GTTTTCCACA ATATTTTAAC ACTGAGGAAA AATTGCTTCT GACCAGCAGT     540
AGAGTTCATC TTTGCCGAGA GCTCACCTGT GAGGGGCTTC TACAAAGGCT TGAGTCTTTG     600
GAGGAAGAGA TCATTTCGAA AGGAGTTAAG CTTGTGATTG TTGACTCCAT TGCTTCTGTG     660
GTCAGAAAGG AGTTTGACCC GAAGCTTCAA GGCAACATCA AAGAAAGGAA CAAGTTCTTG     720
GGCAAAGGAG CGTCCTTACT GAAGTACCTG GCAGGGGAGT TTTCAATCCC AGTTATCTTG     780
ACGAATCAAA TTACGACCCA TCTGAGTGGA GCCCTCCCTT CTCAAGCAGA CCTGGTGTCT     840
CCAGCTGATG ATTTGTCCCT GTCTGAAGGC ACTTCTGGAT CCAGCTGTTT GGTAGCTGCA     900
CTAGGAAACA CATGGGGTCA CTGTGTGAAC ACCCGGCTGA TTCTCCAGTA CCTTGATTCA     960
GAGAGAAGGC AGATTCTCAT TGCCAAGTCT CCTCTGGCTG CCTTCACCTC CTTTGTCTAC    1020
ACCATCAAGG GGGAAGGCCT GGTTCTTCAA GGCCACGAAA GACCATAGGG ATACTGTGAC    1080
CTTTGTCTAG TGCTGATTGC ATGTGACTCA TGAAATGAAA CAGGACTGCG CTGCTTGGAA    1140
AAAGGAAACG GAAGCCAACA TAATGAGGAT TAATTGGTTG GTTGCTGTTG AGGTGGTAAC    1200
AGTGATTTCA GACCCGGAAG GTGAAGATGA AGAAGCCTTT ATCCAGTCTC TGGATGCAGA    1260
GGCTAGGGGC TCCACCACCG TGGGATGTCA GCGGCCATCG TAATAATTTG CACTTACACA    1320
AGCACCTTTC AGCCATGCCC CTCAAAGTGG TTCAGCCACA TTAATTAATT AAAGCCCACA    1380
ATCCCCCTAG GGAGAGCAGG AGGGGGACTA ACAAGATTTG TAATTACAGA AGGGAAAATT    1440
TCCGAATAAA GTATTGTTCC GCCAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA    1500
AAAAAAAAAA AAAAAAAAAA AAAAA                                          1525
```

FIG. 1G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100
A BOX
EGAUUYIDTESAFSAERLUEIAESRFPRYFNTEEKLLLTSSKUHLY
150
P
RELTCDEULQRIESLEEEIISKGIKLUILDSUASUURKEFDAQLQG
DNA
200
B BOX
NLKERNKFLAREASSLKYLAEEFSIPUILTNQITTHLSGALASQAD
250
LUSPADDLSLSEGTSGSSCUIAALGNTWSHSUNTRLILQYLDSERR
300
QILIAKSPLAPFTSFUYTIKEEGLULQAYGNS*
350

FIG. 2A

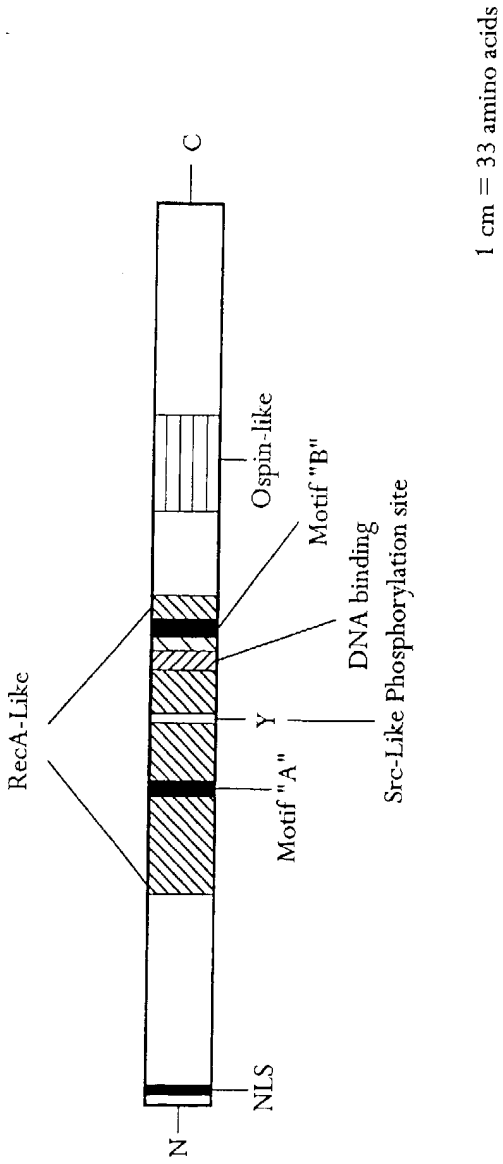
1 cm = 33 amino acids
C
Ospin-like
Motif "B"
DNA binding
Src-Like Phosphorylation site
RecA-Like
Y
Motif "A"
NLS
N

FIG. 2B

```
U.m. 124 LNDARFASSCIVPPTQGYDGNFPGAQCFVYDSDAGSDSDARSSIDAVMHE 173
Human  1 MGSKKLKR...VGLSQELCDRLSRHQILTCQDFLCLSPLELMKVTGLSYR 47
     174 DI.ELPSTFCRPQTPQTHDVARDEHHDGYLCDPKVDHASVARDVLSLGRQ 222
      48 GVHELLCMVSRA..................CAPKMQTAYGIKAQRSADFS 79
     223 RHVFSSGSRELDDLLGGGVRSAVLTELVGESGSGKTQMAIQVCTYAALGL 272
      80 PAFLSTTLSALDEALHGGVACGSLTEITGPPGCGKTQFCIMMSILATL.. 127
     273 VPLSQADDHDKGNNTFQSRTFVRDPIHASTKDDTLSDILQSYGMEPSIGS 322
 128 ..........................PTNMGGLEG............. 136
     323 HRGMGACYITSGGERAAHSIVNRALELASFAINERFDRVYPVCDPTQSSQ 372
     137 ....AVVYIDTESAFSAERLVEIA........ESRFPRYF.......... 164
     373 DADGRRDALLAKAQQLGRRQALANLHIACVADVEALEHALKYSLPGLIRR 422
     165 ...NTEEKLLLTSSKVHLYRELTCDEV..LQRIESLEEEI.......... 199
```

FIG. 2C

```
423 LWSSKRQSGVSREIGVVVVDNLPALFQQDQAAASDIDSLFQRSKMLVEIA 472
          :|::| :|::|.::.:...: .|. : :.| :|.|:|.  |
200 .........ISKGIKLVILDSVASVVRKEFDAQLQ.GNLKERNKFLAREA 239

473 DALKRISAVQWRGASDCGSSAGRAVLVLNHVSDAFGIDKQIARRFVFDSA 522
    ..||                      .: :..| :. :..||. ::
240 SSLK.....................YLAEEFSIPVILTNQITTHL..... 263

573 SGLLASIAPTLAEAVGARELDSACASNDVPLRTLEARTAQLGQTWSNLIN 622
    || ||| |. :..| : .  ::.::|. |        .|.||.|||: :|
264 SGALASQADLVSPADDLSLSEGTSGSSCV.........IAALGNTWSHSVN 305

623 VRVFL....SKTRARICMRDDQAPACEPVRQNTNQRGTASKSLMNTVRKA 668
    .|::|    |. |. :. :.. ||               .|:: |::..
306 TRLILQYLDSERRQILIAKSPLAP...............FTSFVYTIKEE 340

669 AVVINPFGAT 678
    ::|:.::|..
341 GLVLQAYGNS 350
```

FIG. 2D

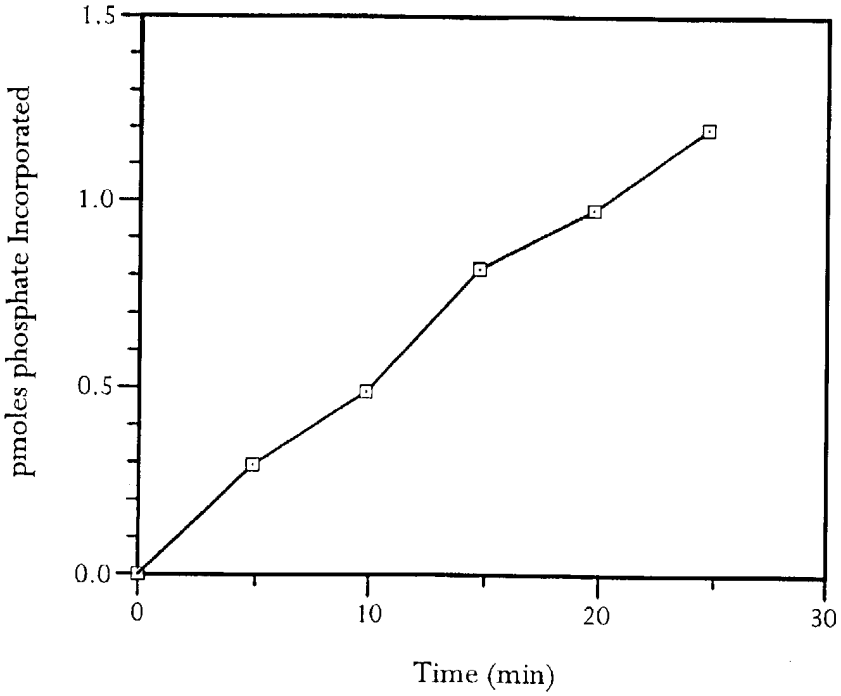

FIG. 3A

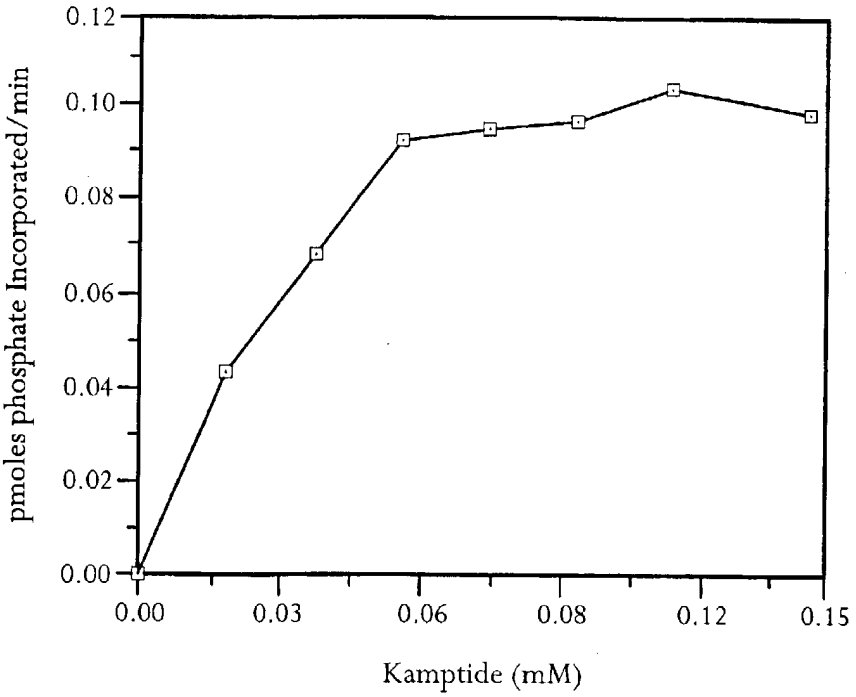
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FIG. 3B

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Cornell Research Foundation
Kimeragen, Inc.

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Gln Thr Ala Tyr Glu Leu Lys Thr Arg Arg Ser Ala His Leu Ser Pro
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Ala Phe Leu Ser Thr Thr Leu Cys Ala Leu Asp Glu Ala Leu His Gly
                85                  90                  95

Gly Val Pro Cys Gly Ser Leu Thr Glu Ile Thr Gly Pro Pro Gly Cys
            100                 105                 110

Gly Lys Thr Gln Phe Cys Ile Met Met Ser Val Leu Ala Thr Leu Pro
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Glu Ser Leu Glu Glu Glu Ile Ile Ser Lys Gly Val Lys Leu Val Ile
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Val Asp Ser Ile Ala Ser Val Val Arg Lys Glu Phe Asp Pro Lys Leu
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Gln Gly Asn Ile Lys Glu Arg Asn Lys Phe Leu Gly Lys Gly Ala Ser
225                 230                 235                 240

Leu Leu Lys Tyr Leu Ala Gly Glu Phe Ser Ile Pro Val Ile Leu Thr
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/21642

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 9/12; C12Q 1/48; A61K 38/51
US CL :435/15, 194; 424/94.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/15, 194; 424/94.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE, BIOSIS, BIOTECHDS, SCISEARCH, HCAPLUS, LIFESCI, NTIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	STURZBECHER et al. p53 is linked directly to homologous recombination processes via RAD51/RecA protein interaction. EMBO J. 04 April 1996, Vol.15, No.8, pages 1992-2002, see the entire article specially figure 3 and page 1997.	1-4,6-7, 9-10,12-16,18 --------- 5,8,11,17
Y	BJORBAEK et al. Divergent functional roles for p90 kinase domains. J. Biol. Chem. 11 August 1995, Vol. 270, No. 32, pages 18848-18852, see the entire article specially the abstract.	5,8,11,17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
18 NOVEMBER 1999	09 DEC 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MARYAM MONSHIPOURI Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/21642

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RICE et al. Isolation of human and mouse genes based on homology to REC2, a recombinational repair gene from the fungus Ustilago maydis. Proc. Natl. Acad. Sci. U.S.A. 10 Jul 1997, Vol.94, Pages 7417-7422, see HsLIM15 in Figure 1.	19-20